Characterization of Early, Chitin-Induced Gene Expression in *Arabidopsis*

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Three genes (i.e., a zinc finger protein, a lectin-like protein, and *AtMPK3*), previously shown to respond to chitin elicitation in microarray experiments, were used to examine the response of *Arabidopsis* spp. to chitin addition. Maximum induction for all three genes was found upon addition of crab-shell chitin at 100 mg per liter. Threefold induction was found with a chitin concentration as low as 10^{-4} mg per liter. The specificity of this response was examined using purified chitin oligomers (degree of polymerization = 2 to 8). The larger chitin oligomers (hexamer to octamer), were most effective in inducing expression of the three genes assayed. Gene induction was observed after the addition of 1 nM chitin octamer. The protein kinase inhibitors staurosporine and K252a effectively suppressed chitin-induced gene expression, while the protein phosphatase inhibitors calyculin A and okadaic acid induced the accumulation of mRNA in the absence of chitin. The phosphorylation event necessary for transmission of the chitin signal was completed within the first 20 min of chitin addition. The level of chitin-induced gene expression of the lectin-like protein and *AtMPK3* was not significantly changed in mutants blocked in the jasmonic acid (*JA*, *jar1*)-, ethylene (*ein2*)-, or salicylic acid (*SA*, *pad4*, *npr1*, and *eds5*)-dependent pathway. In contrast, expression of mRNA for the zinc finger protein was reduced in the mutants affected in the *JA*- or *SA*-dependent pathway.

Plants possess a variety of defense mechanisms against pathogens such as fungi, bacteria, viruses, nematodes, and insects. Race-specific resistance is mediated through a gene-for-gene recognition event involving a plant host resistance (*R*) gene and the corresponding pathogen avirulence (*avr*) gene (Ellis et al. 2000; Martin 1999). Non-race resistance is genetically more complex and can occur in plants in response to a variety of pathogens. Non-race resistance can be triggered in plants by recognition of specific elicitors, such as glucan, chitin/chitosan, or peptide/glycopeptide (Ebel and Cosio 1994). Chitin (β -1,4 linked *N*-acetylglucosamine) oligomers, presumably released from fungal cell walls, can act as elicitors of a plant defense response against invading fungal pathogens (Stacey and Shibuya 1997). For example, Yamada and associates (1993) found that purified chitin fragments (degree of polymerization [dp] = 7 to 8) could induce phytoalexin formation in rice tissue. Chitin treatment of rice also induced transient

membrane depolarization (Kuchitsu et al. 1993a), ion efflux, cytoplasmic acidification (Kuchitsu et al. 1997), transient generation of reactive oxygen species (ROS) (Kuchitsu et al. 1995), protein phosphorylation (Kuchitsu et al. 1993b), jasmonic acid biosynthesis (Nojiri et al. 1996), and transient expression of several unique “early responsive” genes (Minami et al. 1996b). Chitin also induced expression of typical defense-related genes, such as phenylalanine ammonia lyase (*PAL*) and chitinase (Nishizawa et al. 1999) in suspension-cultured rice cells. Chitin oligomers were shown to induce medium alkalinization, ROS generation (Day et al. 2001), and mRNA expression (Minami et al. 1996a) in suspension-cultured, soybean cells. Similar to the situation in rice, soybean responded most strongly to the larger chitin oligomers tested (dp = 7 to 8) (Day et al. 2001). Chitin oligomers were shown to induce various defense-related cellular responses in tomato (Felix et al. 1993), wheat (Barber et al. 1989), oat (Ishihara et al. 1996), and melon (Roby et al. 1987). Some of these responses, such as changes in ion fluxes and the generation of ROS, occur very rapidly and likely involve post-translational regulation. Other responses, such as the accumulation of *PAL*, chitinase, and other proteins, involve induction of gene expression.

The availability of powerful genetic tools and the recently completed genome sequence make the genus *Arabidopsis* one of the best models for plant defense response research. Only a few investigators have examined the response of *Arabidopsis* spp. to chitin elicitation. Transgenic *Arabidopsis* seedlings carrying an *ATL2-GUS* fusion revealed that the expression of *ATL2*, a RING-H2 protein, was rapidly induced after exposure to chitin (Salinas-Mondragon et al. 1999). Rapid induction of another member of the *ATL* family also was observed under the same conditions. The expression of another gene, *STP4* (sugar transport protein 4), also was induced rapidly in suspension-cultured *Arabidopsis* cells treated with chitin (Tuernit et al. 1996).

Recently, we identified 71 expressed sequence tags (EST), representing 61 genes, that responded (more than threefold) to chitin elicitation using DNA microarrays consisting of 2,375 *Arabidopsis* EST (Ramonell et al. in press). Differential gene expression was seen as early as 10 min posttreatment. This study showed that chitin is an efficient and rapid elicitor of gene expression in *Arabidopsis* spp. and, therefore, this plant is a useful model to further explore the role of chitin in eliciting a plant defense response.

In order to better define the response of *Arabidopsis* spp. to chitin, we focused our experiments on three genes, encoding a zinc finger protein (*At1g27730*), a lectin-like protein (*At3g16530*), and *AtMPK3* (*At3g45640*, encoding a MAP kinase), which were previously shown in the DNA microarray

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experiments to be rapidly and strongly induced upon chitin addition. The data indicate that chitin is a potent elicitor of gene expression in *Arabidopsis* spp. and this induction likely involves a rapid protein phosphorylation step. Surprisingly, induction of mRNA encoding the lectin-like protein and *AtMPK3* was not affected in mutants blocked in the jasmonic acid (JA)-, salicylic acid (SA)-, or ethylene-dependent defense pathways. In contrast, mutants blocked in either the JA or SA pathway did reduce chitin induction of mRNA for the zinc finger protein. These data suggest that chitin elicitation of gene expression in *Arabidopsis* spp. may be complex and involve unique signal transduction pathways.

RESULTS

Sensitivity of *Arabidopsis* spp. to chitin elicitation.

Previous DNA microarray experiments showed that *Arabidopsis* seedlings responded strongly to the addition of crab shell chitin at 100 mg/liter (Ramonell et al. in press). The strongest response was seen between 30 min and 3 h after chitin addition. Among the various genes responding to chitin addition, mRNA for *AtMPK3*, a lectin-like protein, and a zinc finger protein accumulated to a high level within 30 min of elicitor addition. Therefore, we chose to use these genes as molecular tools to further examine the response of *Arabidopsis* spp. to chitin elicitation. In order to determine the sensitivity of *Arabidopsis* spp. to chitin elicitation, 14-day-old hydroponic-growing plants were treated for 30 min with chitin (10^{-4} to 100 mg/l) and the level of mRNA accumulation was measured by Northern blot hybridization (Fig. 1). Maximum induction was found at 100 mg/l for the three genes tested. Weaker induction was seen at the lower concentrations but induction was still greater than threefold even at 10^{-4} mg/l. Therefore,

Fig. 1. Induction of zinc finger protein, lectin-like protein, and *AtMPK3* mRNA by chitin addition. *Arabidopsis* seedlings were treated for 30 min with crab-shell chitin at concentrations ranging from 10^{-4} to 10^2 mg per liter or with water (con). Total RNA was isolated and used for Northern blot analysis. The *actin-2* gene was used as a loading control. The fold induction of each gene for a given treatment was determined by the following calculation: (total counts of gene in treated sample/total counts of *actin-2* in treated sample)/(total counts of gene in control sample/total counts of *actin-2* in control sample). The triangle, dot, and diamond lines represent the fold induction of zinc finger protein, lectin-like protein, and *AtMPK3* mRNA, respectively. Experiment was repeated multiple times with similar results.

chitin appears to be a potent inducer of gene expression in *Arabidopsis* spp.

Chitin oligomers induce gene expression in *Arabidopsis* spp. in a size-dependent manner.

The chitin used for the microarray experiments was acid-hydrolyzed, crab-shell chitin that likely contained a diverse mixture of different chitin oligomers as well as undefined impurities. In order to better define the specific chitin molecule that elicits gene expression in *Arabidopsis* spp., we analyzed the expression of the three genes in response to different, purified chitin oligomers. *Arabidopsis* seedlings were incubated with different chitin oligomers (dp = 2 to 8, 10^{-6} M) for 30 min and gene expression was analyzed by Northern blot hybridization (Fig. 2). The larger chitin oligomers (dp = 6 to 8) were most effective in inducing the expression of the three genes assayed. These results are very similar to the previously reported responses of rice and soybean to chitin elicitation (Day et al. 2001; Shibuya et al. 1996).

The response of *Arabidopsis* seedlings to varying concentrations of the purified chitin octamer (10^{-6} to 10^{-12} M) is shown in Figure 3. Maximum induction was found at 10^{-6} and 10^{-7} M for the three genes tested. However, mRNA accumulation for the strongly expressed zinc finger protein was obvious at 10^{-9} M. Again, these data compare favorably to data from rice and soybean where chitin octamer binding to plasma membrane preparations showed K_d values of 29 and 35 nM, respectively (Day et al. 2001; Shibuya et al. 1996).

Protein phosphorylation is required for early chitin-induced gene expression.

Protein phosphorylation cascades are known to be involved in plant intracellular signaling pathways (Torii 2000). For example, chitin elicitor addition was shown to induce protein phosphorylation in suspension-cultured rice cells (Kuchitsu et

Fig. 2. Induction of zinc finger protein, lectin-like protein, and *AtMPK3* mRNA by chitin oligomers. *Arabidopsis* seedlings were incubated with different chitin oligomers (degree of polymerization = 2 to 8, 10^{-6} M), crab-shell chitin at 100 mg/liter (chi), or water (con) for 30 min. Total RNA was isolated and used for Northern blot analysis. The *actin-2* gene was used as a loading control. The fold induction of each gene for a given treatment was determined by the following calculation: (total counts of gene in treated sample/total counts of *actin-2* in treated sample)/(total counts of gene in control sample/total counts of *actin-2* in control sample). The horizontal, vertical, and slant line bars represent the fold-induction of zinc finger protein, lectin-like protein, and *AtMPK3* mRNA, respectively. Experiment was repeated multiple times with similar results.

al. 1993b). To examine the involvement of protein phosphatases and kinases in the *Arabidopsis* chitin response, various inhibitors were added and their effect on chitin induction of gene expression tested. Calyculin A and okadaic acid, two potent inhibitors of serine/threonine phosphatases type 1 and type 2A from both animals and plants, did not inhibit chitin induced gene expression at 1 μ M (Fig. 4). On the contrary, calyculin A induced mRNA accumulation for the three genes tested in the absence of chitin (Fig. 4). Lower-level induction of mRNA accumulation was observed for okadaic acid, but the induction of mRNA for the zinc finger protein and lectin-like protein was still obvious. Consistent with these results, staurosporine and K252a, two potent inhibitors of plant and animal serine/threonine protein kinases, effectively suppressed chitin-induced gene expression (Fig. 4). Chitin-induced mRNA accumulation was greatly reduced, but not completely blocked in the presence of 5 to 10 μ M staurosporine (Fig. 5A), whereas it was completely blocked in the presence of 0.5 μ M or 5 μ M K252a for lectin-like protein and *AtMPK3* or zinc finger protein, respectively (Fig. 5B). These data suggest the likely involvement of protein phosphorylation in early, chitin-induced gene expression. The above inhibitors were added dissolved in dimethyl sulfoxide (DMSO); therefore, we tested this solvent alone as a control. DMSO was able to induce low-level gene expression (Fig. 4). Kodama and associates (2000) reported similar induction of the *Arabidopsis* fatty acid desaturase gene by DMSO.

We examined the timing of the phosphorylation event necessary for chitin response by adding staurosporine (10 μ M) together with crab-shell chitin (100 mg/l) or at various times after chitin addition (Fig. 6). When staurosporine was simultaneously added with chitin, mRNA induction for the zinc finger protein, lectin-like protein, and *AtMPK3* was repressed 43, 42,

Fig. 3. Induction of zinc finger protein, lectin-like protein, and *AtMPK3* mRNA by chitin octamer. *Arabidopsis* seedlings were treated for 30 min with chitin octamer at concentrations ranging from 10^{-6} to 10^{-12} M or with water (con). RNA was isolated and used for Northern blot analysis. The *actin-2* gene was used as a loading control. The fold induction of each gene for a given treatment was determined by the following calculation: (total counts of gene in treated sample/total counts of *actin-2* in treated sample)/(total counts of gene in control sample/total counts of *actin-2* in control sample). The triangle, dot, and diamond lines represent the induction fold of zinc finger protein, lectin-like protein, and *AtMPK3* mRNA, respectively. Experiment was repeated multiple times with similar results.

or 33%, respectively. If the staurosporine was added at times subsequent to chitin addition, the repressive effect was reduced. Addition of staurosporine 20 min after chitin addition gave no repression. These results indicate that the essential phosphorylation event for chitin elicitation is complete within 20 min of elicitor treatment.

Role of the various plant defense pathways in chitin elicitation.

Disease resistance in *Arabidopsis* spp. is regulated by multiple signal transduction pathways in which SA, JA, and ethylene function as key signaling molecules (Dong 2001; Glazebrook 2001). One advantage of using *Arabidopsis* spp. to study chitin elicitation is the availability of mutants blocked in the various defense pathways. Mutants that affect the JA-dependent pathway (*jar1*), ethylene-dependent pathway (*ein2*), or SA-dependent pathway (*pad4*, *eds5*, *npr1*, and *cpr1*) were examined for their ability to respond to chitin elicitation. All of the mutants used in this experiment, with the exception of *npr1* (No-o background), are in the Col-o background. Therefore, mRNA from wild-type No-o was used as the control for *npr1* Northern blots, while mRNA from Col-o plants was used for other mutants. The data clearly show that none of the mutants were completely blocked in their ability to rapidly (i.e., at 30 min) induce mRNA expression of the three genes in response to chitin addition (Fig. 7). Moreover, the level of chitin-induced gene expression for the lectin-like protein and *AtMPK3* was not significantly changed in any of the mutants tested. These data suggest that chitin response may involve a unique signal transduction pathway that culminates in the expression of the lectin-like protein and *AtMPK3*. This conclusion is consistent with our previous DNA microarray experiments that failed to show a correlation between genes induced upon chitin addition and other treatments (Ramonell et al. in press).

In contrast to the situation with the lectin-like protein and *AtMPK3*, the level of mRNA encoding the zinc finger protein was decreased approximately one-third in the *jar1* mutant compared with the wild-type Col-o upon chitin treatment (Fig. 7). The chitin-induced expression level of the zinc finger protein mRNA also was significantly decreased in *pad4* and *eds5* mutants. Consistent with these results, the background expression level of the zinc finger protein was much higher in the *cpr1* mutant than in the wild-type or other mutants. These results suggest that there might be crosstalk between the chitin signaling pathway and the JA- and SA-

Fig. 4. Effect of protein kinase and phosphatase inhibitors on chitin-induced mRNA accumulation. *Arabidopsis* seedlings were incubated for 30 min, with or without crab-shell chitin (0.1 mg per liter), in the absence or presence of various protein kinase inhibitors (10 μ M staurosporine [Stau] or 10 μ M K252a) or phosphatase inhibitors (1 μ M calyculin A [Cal A] or 1 μ M okadaic acid [OA]). RNA was isolated and used for Northern blot analysis. The *actin-2* gene was used as a loading control. Experiment was repeated three times with identical results.

dependent pathways. Moreover, the difference in response between the zinc finger protein and the lectin-like protein and *AtMPK3* clearly indicates an unexplained complexity in the mechanism of *Arabidopsis* spp. response to chitin.

Fig. 5. Staurosporine and K252a inhibition of chitin-induced mRNA accumulation. *Arabidopsis* seedlings were incubated for 30 min., with or without (con) crab-shell chitin (0.1 mg per liter), in the absence or presence of **A**, Staurosporine (Stau) or **B**, K252a at the indicated concentrations. RNA was isolated and used for Northern blot analysis. The *actin-2* gene was used as a loading control. The fold induction of each gene for a given treatment was determined by the following calculation: (total counts of gene in treated sample/total counts of *actin-2* in treated sample)/(total counts of gene in control sample/total counts of *actin-2* in control sample). The triangle, dot, and diamond lines represent the induction fold of zinc finger protein, lectin-like protein, and *AtMPK3* mRNA, respectively. Experiment was repeated multiple times with similar results.

DISCUSSION

Previous studies on chitin elicitation in plants were mainly based on suspension-cultured rice, soybean, or tomato cells (Day et al. 2001; Felix et al. 1993; Kuchitsu et al. 1993a, 1993b, 1995, 1997; Nojiri et al. 1996; Yamada et al. 1993). In contrast, our studies involved chitin treatment of whole seedlings. The rapid response of *Arabidopsis* spp. to low concentrations of elicitor suggests that chitin is a potent inducer of gene expression in this plant.

Oligomer size plays a definitive role in the induction of plant defense responses (Hadwiger 1999). Yamada and associates (1993) showed that the induction of phytoalexin formation in suspension-cultured rice cells was strictly dependent on the size of the chitin fragments. Larger chitin fragments, such as hexamer–octamer, could induce phytoalexin biosynthesis even at nanomolar concentration, whereas smaller fragments, such as dimer–trimer, showed almost no activity. Similar results were found in soybean. For example, inhibition analysis of chitin binding showed that the larger chitin oligomers (hexamer–octamer), were better inhibitors of ligand binding to soybean plasma membranes isolated from suspension cultured cells (Day et al. 2001). Soybean suspension-cultured cells showed a binding preference for larger (dp = 8) chitin oligomers with a K_d value of 35 nM (Day et al. 2001), while the K_d value for rice and tomato cells was 29 nM (Shibuya et al. 1996) and 28 nM (Baureithel et al. 1994), respectively. In our experiments, gene induction could be observed after the addition of as little as 1 nM of chitin octamer. These results compare favorably with those from soybean, rice, and tomato. Therefore, *Arabidopsis* spp. likely possess a conserved chitin recognition system that is found in most, if not all, plants.

The interaction of hevein domain (chitin-binding motif) proteins with chitin oligomers (dp = 2 to 8) was studied using isothermal titration calorimetry (ITC), ultracentrifugation, and nuclear magnetic resonance (Asensio et al. 2000). For chitin fragments up to dp = 4, the carbohydrate length was too short to allow the binding of two hevein moieties. Five to seven GlcNAc units in the ligand were required to exhibit all the possible protein–sugar interactions. These biochemical studies may explain why only the larger chitin oligomers are active elicitors in rice, soybean, and *Arabidopsis* spp.

Inhibitors of protein kinases and phosphatases are useful tools to investigate the role of phosphoproteins in signal transduction. This pharmacological approach repeatedly has demonstrated that changes in protein phosphorylation regulate diverse cellular processes in eukaryotes, including some plant defense responses (Romeis 2001). Kuchitsu and associates (1993b) showed that a chitin elicitor could induce protein phosphorylation in suspension-cultured rice cells. Similarly, addition of protein kinase inhibitors suppressed the induction of rice EL1, EL2, and PAL transcripts by N-acetylchitoheptaose addition (He et al. 1998). In our experiments, staurosporine and K252a effectively suppressed chitin-induced gene expression in *Arabidopsis* spp., whereas calyculin A and okadaic acid induced the accumulation of mRNA in the absence of chitin. The phosphorylation event necessary for transmission of the chitin signal was completed within the first 20 min of chitin addition. Peck and associates (2001) identified a number of proteins that were phosphorylated within minutes after chitin treatment of *Arabidopsis* tissue culture cells. In our experiments, the inhibitors affected expression of all three genes; therefore, it seems likely that a protein phosphorylation step is required very early in the cellular response to chitin elicitation. Members of different kinase subfamilies, such as calcium-dependent protein kinases (CDPKs) and MAP kinases, play an essential role in

the plant defense response (Romeis 2001). Changes in cytoplasmic calcium concentration are one of the earliest cellular responses to stress stimuli and CDPKs may function as sensors for this change (Harmon et al. 2000). Transcript accumulation of NtCDPK1 from tobacco was reported upon chitin elicitation (Yoon et al. 1999). Interestingly, a CDPK (At3g57530) was transiently induced more than twofold upon chitin treatment during our previous DNA microarray experiments (Ramonell et al. in press). This or other protein kinases may be involved in the chitin response pathway.

Oligosaccharide elicitors are one of the external signals that elicit the plant's defense response against pathogens. Four major classes of elicitor-active oligosaccharides have been identified: oligoglucan, oligochitin, and oligochitosan (derived from fungal cell walls); and oligogalacturonide (derived from plant cell walls) (Ebel 1998). Oligosaccharides are relatively immobile in plants and are considered to be localized signals (Doares et al. 1995a). The activation of defensive genes by oligosaccharides was proposed to occur via the octadecanoid pathway (Doares et al. 1995b; Farmer and Ryan 1992; Mueller et al. 1993). In this pathway, linolenic acid, released from membrane phospholipids by phospholipase, is converted to 12-oxophytodienoic acid and JA, leading to the transcriptional activation of defensive genes. Nojiri and associates (1996) showed that chitin treatment of rice cells resulted in transient production of JA. Consistent with these reports, the level of mRNA encoding the zinc finger protein was decreased approximately one-third in the *jar1* mutant compared with the wild-type Col-o upon chitin treatment (Fig. 7). These data suggest the involvement of the JA-dependent pathway in chitin signaling in *Arabidopsis* spp. The level of the mRNA for the zinc finger protein was reduced in the chitin-treated *pad4* and *eds5* mutants, elevated in the untreated *cpr1* mutant, and unchanged in the *npr1* mutant relative to wild type. PAD4, EDS5, and CPR1 are thought to act upstream of NPR1 in the SA signal transduction pathway (Dong 2001; Glazebrook 2001); therefore, these results suggest that PAD4, EDS5, and CPR1 could play a role both in chitin induction of the zinc finger protein mRNA level and in the signaling leading to the SA and NPR1. SA previously was shown to be a potent inhibitor of JA-dependent defense responses (Doares et al. 1995a; Gupta et al. 2000; Penninckx et al. 1996). There is also evidence for inhibition of SA action by JA (Rao et al. 2000). Therefore, the general view is that the SA- and JA-dependent pathways are antagonistic. However, Schenk and associates (2000) reported DNA microarray experiments comparing *Arabidopsis* spp. treated with the fungal pathogen *Alternaria brassicicola*, SA, methyl-jasmonate (MJA), or ethylene. In this report, the largest number of both co-induced and co-repressed genes was observed for the SA and JA treatments. Similarly, van Wees and associates (2000) showed that simultaneous activation of SA- and JA-dependent pathways could lead to an elevated level of protection against pathogen attack. These data suggest that SA and JA signaling pathways may share common activating signals. Our data for expression of the zinc finger protein mRNA suggests that chitin is one such signal that involves both the JA- and SA-dependent pathways.

In contrast to the situation with the zinc finger protein, the level of chitin-induced gene expression for the lectin-like protein and *AtMPK3* was not significantly changed in any of the mutants tested (Fig. 7). These data are consistent with our previous comparison of DNA microarray results from treatment of *Arabidopsis* spp. with chitin, the fungal pathogen *Alternaria brassicicola*, SA, MJA, or ethylene (Schenk et al. 2000; Ramonell et al. in press). No general correlation was found be-

tween the chitin response and other treatments. For example, of the 39 genes induced or repressed by more than 2.5-fold (at 30 min) by chitin treatment, only 13 responded (within 24 h) to MJA treatment. However, direct comparison at 24 h post treatment showed that none of the genes showed a similar expression profile to chitin and MJA addition. These data suggest that chitin response involves a signal transduction pathway other than the JA-, SA-, or ethylene-dependent pathways. Therefore, our data, comparing the response of the zinc finger protein to that of the lectin-like protein or *AtMPK3*, reveal an unexpected complexity. Apparently, the response of certain genes, as previously predicted, to chitin involves aspects of the JA- and SA-dependent pathways. In contrast, clearly, other genes respond directly to chitin addition through a unique pathway. The initial activation of both of these response pathways appears to require a rapid protein phosphorylation step. Access to the powerful molecular and genetic tools available in *Arabidopsis* spp. should allow the eventual elucidation of the full chitin response mechanism.

MATERIALS AND METHODS

Chemicals.

Crab-shell chitin was purchased from Sigma-Aldrich (St. Louis) and prepared in ddH₂O as a 100 mg/ml stock solution. Chitin oligomers (dp = 2 to 6) were purchased from Seikagaku Corporation (Tokyo) and prepared in ddH₂O as 1-mM stock solutions. Chitin oligomers (dp = 7-8) were a generous gift from Dr. Naoto Shibuya, National Institute of Agrobiological Resources, Tsukuba, Japan. Staurosporine, K252a, calyculin A, and okadaic acid were obtained from Calbiochem (San

Fig. 6. Effect of the time of addition on staurosporine repression of chitin-induced mRNA accumulation. *Arabidopsis* seedlings were incubated with crab-shell chitin at 100 mg per liter and supplied with 10 μ M staurosporine at the indicated times. The seedlings were harvested 30 min after addition of chitin (time zero). Total RNA was isolated and used for Northern blot analysis. The *actin-2* gene was used as a loading control. The induction level in the NS (no staurosporine addition) sample was set as 100%. The relative induction level of each gene in the presence of staurosporine was determined by the following calculation: (total counts of gene in staurosporine-added sample/total counts of *actin-2* in staurosporine-added sample)/(total counts of gene in NS sample/total counts of *actin-2* in NS sample) $\times$ 100%. The triangle, dot, and diamond lines represent the induction level of zinc finger protein, lectin-like protein, and *AtMPK3* mRNA, respectively. Experiment was repeated multiple times with similar results.

Diego) and dissolved in DMSO as 200-, 500-, 50-, and 100- μ M stock solutions.

Plant materials and growth conditions.

Seed of wild-type *Arabidopsis thaliana* ecotype Col-O and No-O, mutant *npr1-5* (Shah et al. 1997), *cpr1-1* (Bowling et al. 1994), *pad4-1* (Glazebrook et al. 1997), *eds5-1* (Glazebrook et al. 1996), *jar1-1* (Staswick et al. 1992), and *ein2-26* (Alonso et al. 1999) were surface sterilized using 75% ethanol followed by 50% bleach. Approximately 30 seeds of each line were placed in 50-ml Falcon tubes containing 10 ml of liquid Murashige and Skoog medium (Sigma-Aldrich) containing 2% dextrose. These tubes were incubated with gentle shaking for 14 days under constant illumination ($125 \mu\text{mol m}^{-2} \text{s}^{-1}$) at 23°C.

Chitin treatments.

Plant seedlings were grown for 14 days as described above. Crab-shell chitin or individual chitin oligomers (dp = 2 to 8) were added to give the expected final concentrations. Staurosporine, K252a, calyculin A, or okadaic acid were added to the concentrations indicated in the text. The control tubes received an equivalent amount of sterile ddH₂O or DMSO, as required for each treatment. Falcon tubes were incubated for 30 min after chitin addition. Whole seedlings from three tubes for each treatment were combined, the medium drained, and the seedlings rapidly frozen in liquid nitrogen.

RNA isolation, reverse-transcription polymerase chain reaction, and Northern blot analysis.

Total RNA was isolated from the frozen seedlings with Trizol Reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's protocol. RNA samples were treated with RQ1 DNase (Promega, Madison, WI, U.S.A.). First strand cDNA

synthesis was primed with oligo (dT)₁₅ primer (Promega) according to the manufacturer's instructions using M-MLV reverse transcriptase (Promega). An aliquot of the reverse transcription mixture was used as template for polymerase chain reaction (PCR). PCR was performed using standard conditions with the following gene specific primers: zinc finger protein forward = 5'-TGTCACGCAACTTCCTTCT-3', zinc finger protein reverse = 5'-TGGTGTCACTTTATGCTTATTC-3', lectin-like protein forward = 5'-ACAATGCAGATTCACAAACTC-3', lectin-like protein reverse = 5'-GCAAACGATACCTAGCCAA-3', *AtMPK3* forward = 5'-ATGAACACCGGCGGTGGCC-3', *AtMPK3* reverse = 5'-GGCATTACGGGGCTGCTG-3', *actin-2* forward = 5'-GTTGGTGTATGAAGCACAATCCAAG-3', and *actin-2* reverse = 5'-CTGGAACAAGACTTCTGGGCATCT. PCR products corresponding to DNA fragments of the expected size were cloned into pGEM-Teasy Vector (Promega). The resulting fragment first was confirmed by restriction enzyme digestion and then by DNA sequencing. The insert DNA of each clone subsequently was used as a probe for Northern blot analysis. Total RNA (10 μ g/lane) was separated on a denaturing, formaldehyde, 1% agarose gel and vacuum blotted onto Zeta probe Nylon membrane (Bio-Rad Laboratories, Hercules, CA, U.S.A.). In all cases, gene-specific probes were labeled to a specific activity of 10^8 cpm of DNA per microgram with random primer labeling (Promega). Hybridization and washing were performed at high stringency according to the manufacturer's protocol. All blots were counted for total radioactivity using an Instant Imager (Packard Instrument Co., Meriden, CT, U.S.A.). *Actin-2* was used as a loading control. The fold induction of each gene for a given treatment was determined by the following calculation: (total counts of gene in treated sample/total counts of *actin-2* in treated sample)/(total counts of gene in control sample/total counts of *actin-2* in control sample).

Fig. 7. Chitin-induced gene expression in *Arabidopsis* mutant plants. Wild-type (Col-o or No-o) and mutant (*jar1*, *ein2*, *cpr1*, *pad4*, *eds5*, or *npr1*) seedlings were incubated for 30 min, with or without crab-shell chitin (100 mg/liter). Total RNA was isolated and used for Northern blot analysis. The *actin-2* gene was used as a loading control. The relative gene expression level for a given mutant was determined by the following calculation: (total counts of gene in mutant sample/total counts of *actin-2* in mutant sample)/(total counts of gene in wild-type water-treated sample/total counts of *actin-2* in wild-type water-treated sample). No-o was used as wild-type control for the *npr1* mutant, while Col-o was used for all the other mutants. The white and black boxes represent the relative gene expression level for water-treated sample and chitin-treated sample, respectively. Results from a representative experiment are shown. Experiment was repeated three times with similar results.

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